Cloning, tissue distribution, subcellular localization and overexpression of murine histidine-rich Ca^{2+} binding protein

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Abstract The histidine-rich Ca^{2+} binding protein (HRC) resides in the sarcoplasmic reticulum of muscle and binds Ca^{2+}. Since Ca^{2+} concentrations can regulate gene expression via calcineurin, the mouse homologue of HRC (mHRC) was isolated and characterized. mHRC was detected in muscle progenitor cells, in primary clonal thymic tumors and a tumor cell line, suggesting a broader role for mHRC than in Ca^{2+} storage during muscle contraction. mHRC was present in the perinuclear region of myoblasts. To examine if it can regulate gene expression, mHRC was overexpressed in cells differentiating into cardiac and skeletal muscle. mHRC had no effect on cardiogenesis or myogenesis. Therefore, if mHRC plays a role in the regulation of gene expression during cellular differentiation, it does not appear to be either rate-limiting or inhibitory.

Key words: Sarcoplasmic reticulum; Ca^{2+} binding; Leukemia; Striated muscle

1. Introduction

The sarcoplasmic reticulum plays an important role in the regulation of Ca^{2+} concentrations during muscle contraction and relaxation. Ca^{2+} is released by activation of the ryanodine receptor and pumped back into the lumen of the sarcoplasmic reticulum by the Ca^{2+} ATPase (reviewed in [1]). Several proteins reside in the lumen and are believed to help in regulating this process by binding Ca^{2+}. These proteins include calsequestrin, calreticulin and the histidine-rich Ca^{2+} binding protein (HRC). Calreticulin is expressed ubiquitously while calsequestrin and HRC are highly expressed in adult heart and skeletal muscle [2–6].

The sarcoplasmic reticulum proteins have been shown to play more complex roles than merely binding free Ca^{2+}. Calreticulin has been shown not only to bind and regulate Ca^{2+} in the endoplasmic reticulum (ER)/sarcoplasmic reticulum, but also to be involved in the regulation of gene expression, cell adhesion and protein folding [6]. Overexpression of calreticulin inhibited the retinoic acid-induced differentiation of P19 cells into neurons and thus, a role in regulating gene expression appears biologically relevant [7]. Similarly, overexpression of calsequestrin in murine cardiomyocytes results in cardiac hypertrophy and modulated gene expression [8,9]. These results suggest that Ca^{2+} storage proteins play an important role in regulating gene expression and are consistent with the recent findings that Ca^{2+} concentrations can regulate muscle-specific gene expression via the calcineurin/NFAT pathway [10,11].

HRC, unlike calsequestrin and calreticulin, is a poor-studied protein. It binds zinc, Ca^{2+} and lipoproteins in vitro although the biological significance of these properties is still poorly understood [4,5,12]. HRC is hypothesized to be part of the ryanodine receptor-dihydropyridine receptor complex found in triad structures, which is involved in Ca^{2+} release [13,14]. Given the importance of the Ca^{2+} storage proteins, the mouse homologue of HRC (mHRC) has been cloned and its tissue distribution, subcellular localization and effect on early muscle development have been examined.

2. Material and methods

2.1. Cloning and sequence analysis

In order to isolate a transcript overexpressed in TBLV-induced thymic lymphomas [15], an adult mouse skeletal muscle library was screened at high stringency with a unique 1 kb genomic probe from the TBLV integration locus on the X-chromosome [16]. Three clones were isolated and sequenced on both strands using standard techniques and were found to encode mHRC. mHRC is not located on the X-chromosome, but hybridized to the genomic fragment via GC-rich regions.

2.2. Northern Blot analysis

Total RNA was isolated, Northern blots were performed and probes were prepared as described previously [17]. P19 cells, with and without the expression of myogenin, were harvested while growing in monolayer (day 0), while growing as aggregates in 0.8% DMSO (days 1–4) and following aggregation (days 5 and 6) as described ([18], Ridgeway and Skerjanc, unpublished results). Poly-A+ mRNA was prepared from normal thymus and primary clonal thymic tumors from TBLV-infected mice [16] using the FastTrack kit (Invitrogen, Carlsbad, CA, USA). Multiple tissue Northern blots (MTN blots) containing total RNA from normal human tissues (cat. # 7759-1 and 7760-1) and from eight different human cancer cell lines (cat. # 7757-1) were obtained from Clontech Laboratories.

2.3. Cell culture and DNA transfection

P19 embryonal carcinoma cells and P19 (myogenin) stable cell lines were cultured, differentiated and transfected as described previously ([19,20], Ridgeway and Skerjanc unpublished data). Briefly, P19 cells were aggregated for 4 days in the presence of 0.8% dimethylsulfoxide, prior to plating in tissue culture dishes. C2C12 cells were cultured under growth conditions in 5% fetal calf serum:5% calf serum.

The plasmid construct PGK-HRC contains the pgk-1 promoter driving the full length mHRC. PGK-HRC-myc has six myc-epitopes inserted in frame into the *Kpn*I site at 1151 bp in the acidic repeat region of mHRC. Transient transfections were performed by the calcium phosphate method [21] with either 4 μg PGK-HRC-myc or 4 μg PGK-LacZ on cells growing exponentially on gelatin-coated coverslips. The transfected cells were fixed with 3% paraformaldehyde and membranes were permeabilized using 0.1% Triton X-100/PBS. Immunofluorescence was performed as described previously [22]. Immunofluorescent images were visualized with a Zeiss Axioskop microscope, captured with a Sony 3CCD color video camera, processed using Northern Exposure, Adobe photoshop and Corel Draw software and printed with a dye sublimation phaser 450 Tektronic printer.

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Stable P19 cell lines expressing mHRC were created by transfecting 10 μg PGK-HRC, 1 μg PGK-puromycin, 2.5 μg B17 [23] and 1 μg PGK-LacZ into P19 cells. High expressing P19 cell lines were isolated and examined for their ability to differentiate as described previously [17]. Stable myoblast cell lines expressing mHRC were created by transfecting 10 μg PGK-mHRC, 1 μg PGK-puromycin and 1 μg PGK-LacZ. Myoblasts were selected in puromycin for 1 week, transferred into differentiation media containing 2% horse serum for 4 days and colonies containing differentiated myotubes were counted.

3. Results and discussion

The mHRC [5,24] was cloned (GenBank accession number AF158597) and its sequence analyzed (Fig. 1). The longest clone of mHRC was 2270 bp and encoded a protein of 726 amino acids. mHRC, like the rabbit and human homologues, contains a conserved N-terminal signal sequence (amino acids 1–28), an acidic repeat region (amino acids 213–481), a cysteine repeat region (amino acids 654–700) and a stretch of 13 glutamic acid residues (amino acids 590–602).

The cysteine-rich region contains the highest amino acid homology between the three species, with 81 or 86% homology between mHRC and the rabbit or human homologues, respectively. This region seems to be involved in binding Zn^{2+} and LDL [4,5,12]. Since a prosite scan (Lasergene software, DNAStar, Madison, WI, USA) identifies it as an EGF-like cysteine repeat domain, this region may be involved in protein-protein interactions. The amino-terminal domain is less conserved, 66 or 56% homology between mHRC and the rabbit or human homologues, respectively. This domain appears to encode a hydrophobic signal sequence for translocation into the ER.

The acidic repeat region is the least conserved, but a careful examination of the acidic repeat sequences revealed a consensus sequence for two types of repeats, type A and B, described previously [5,24] (Fig. 2). The consensus sequence for the type A human and rabbit repeats both had similarity indices of 67% to the consensus sequence for the mouse type A repeat, by a Lipman-Pearson protein alignment [25]. The consensus sequences for the type B human or rabbit repeats had similarity indices of 44 or 36%, respectively, to the mouse consensus sequence. The overall pattern of repeats was more similar for mouse and human than for rabbit and human. These acidic repeats are believed to bind Ca^{2+} with a low affinity [5].

In agreement with results found previously [5], an examination of the expression pattern of HRC in adult human tissues showed high levels of HRC in heart and skeletal muscle (Fig. 3A). No mHRC expression was detected in colon or small intestine, tissues rich in smooth muscle. A more in-depth analysis of the expression pattern showed high levels of HRC transcripts in primary clonal thymic tumors (Fig. 3B) and in the chronic myelogenous leukemia cell line, K-562 (Fig. 3C). Therefore, mHRC is expressed in specific types of cancer cells

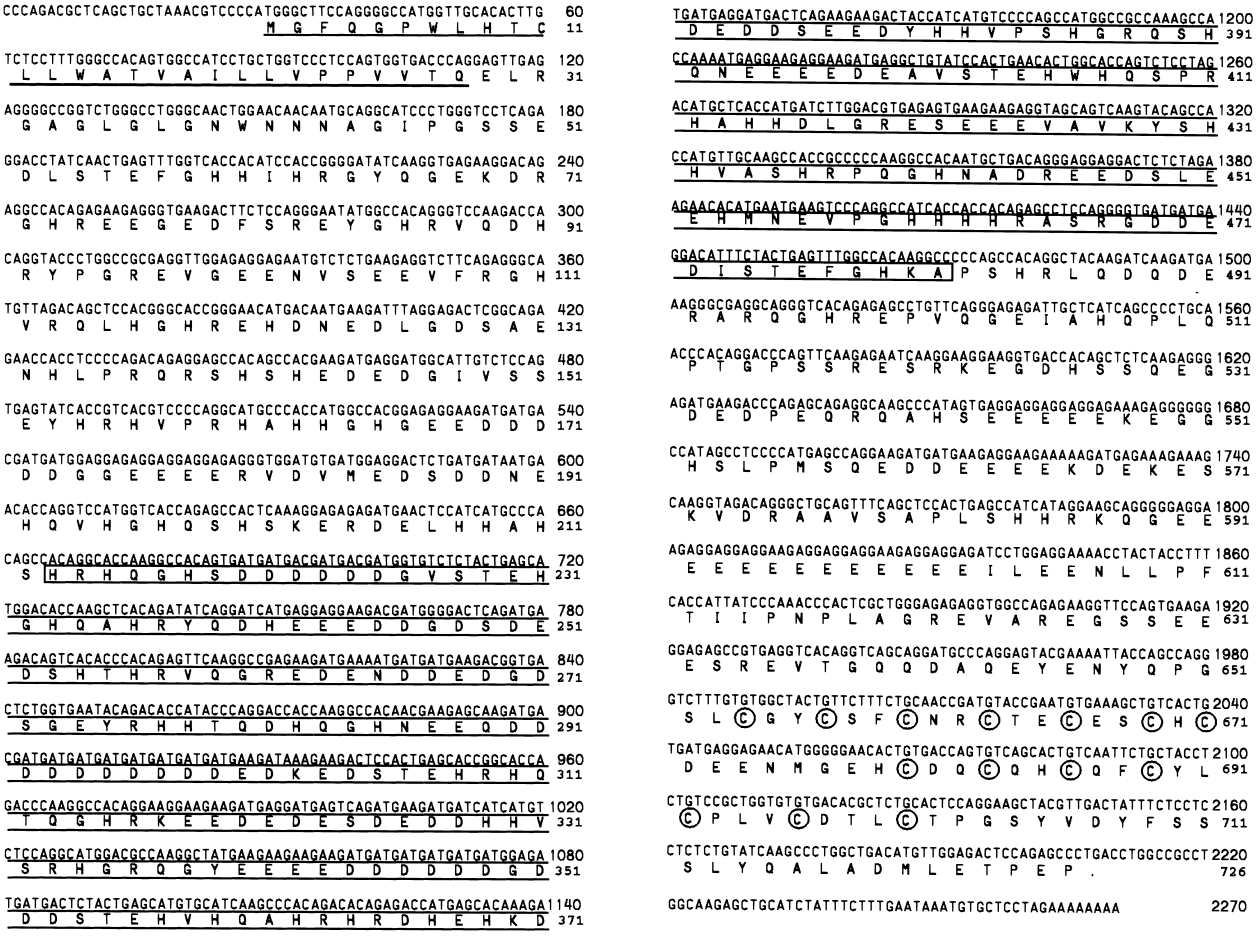

Fig. 1. The nucleotide (top line) and amino acid (bottom line) sequences of murine HRC. The N-terminal signal sequence is underlined, the acidic repeat region is marked by the open box and the cysteine residues in the cysteine repeat region are circled.

Type A Repeats

	aa RESIDUE
-HRHQGHS-------DDDDDDGV------STEHGHQA	213-235
---HQGH---NEEQDDDDDDDDDDEDKEDSTEHRHQT	282-312
RHGRQG--Y--EEEEDDDDDDGDDD----STEHVHQA	333-361
-HGRQ--SHQNEEEEDEA------V----STEHWHQS	386-409
HHHHRA-SRG---DDEDI----------STEFGHKA	460-481
	Consensus
xHxHQG-S--NEEEDDDDDDDxxxxxxxxSTEHx-HQA	**mouse**
-HRHQGHE---EDDEDDDDxxxxxxxxxVSTEYG-HQA	**human**
-HRHRGHR---EEEDEDDDDE------GDSTESDRHQA	**rabbit**

Type B Repeats

	aa RESIDUE
HRYQDH---EEEDDGDSDED-S-HT	236-255
HRVQGREDENDDEDGDSGEYR-HHTQD	256-281
HRHQTQGHRKEEDEDESDEDD-HHV-S	308-332
HRHRDHEH-KDDEDD-SEEDY-HHVPS	362-385
SPRHAHHDLGRESEEEVAVKYSHHVAS	410-435
HRPQGH--NADREED-SLEEHMNEVPG	436-459
	Consensus
HRxQxHxDxxDEEEDESxExxxHHVxS	**mouse**
HRHQXHG-xxxEEDEDVSxEHHHHXPS	**human**
HRHQGHGKEGEEEGxSxEDEHHHHVPx	**rabbit**

Repeat Pattern

mouse	**ABBABABABBA**
human	**BBBABABABA**
rabbit	**AAAAAAAAABBBB**

Fig. 2. Acidic repeats conserved across species in HRC. The type A and B repeats were used as templates to determine repeats found within mHRC. The murine type A and B amino acid sequences and residues are listed along with the overall consensus sequence for mouse, human and rabbit. The overall repeat patterns are shown at the bottom of the figure.

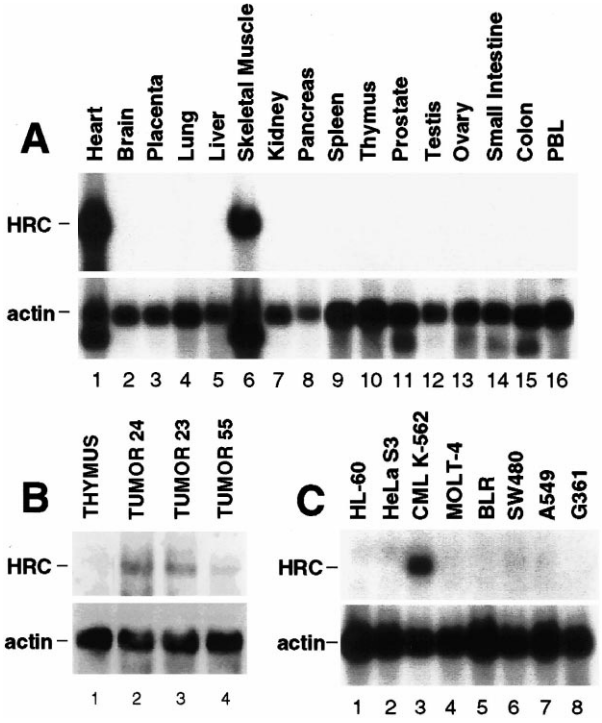

Fig. 3. HRC is overexpressed in TBLV-induced T-cell thymic lymphomas and a chronic myelogenous leukemia (CML) line. (A) MTN blots from Clontech were probed with human HRC (hHRC). The blots contain total RNA from the various normal human tissues indicated. PBL: peripheral blood leukocytes. (B) A Northern blot of poly-A+ RNA from normal thymus (lane 1) and tumors from three separate mice infected with TBLV (lanes 2–4) was probed with mHRC. (C) A MTN blot (Clontech) containing total RNA from eight cancer cell lines was probed with hHRC. HL-60: promyelocytic leukemia cell line; CML K-562: chronic myelogenous leukemia cell line; MOLT-4: lymphoblastic leukemia cell line; BLR: Burkitt's lymphoma Raji cells; SW 480: colorectal adenocarcinoma cell line; A549: lung carcinoma cell line; G361: melanoma cell line. All blots are standardized to actin.

and is not limited to mature muscle fibers. In addition, an examination of HRC expression during the differentiation of P19 cells into cardiac muscle indicated that HRC was expressed on day 4 of differentiation, a full day before the first muscle-specific structural gene, cardiac α-actin, is expressed (Fig. 4). Using a P19 cell line overexpressing myogenin (Ridgeway, A.G. and Skerjanc, I.S., unpublished data) to examine differentiation into skeletal muscle, HRC was present on day 3, 3 full days before the skeletal muscle-specific marker, myosin light chain (MLC) 1/3, was expressed (Fig. 4). Therefore, mHRC is expressed very early during cardiac and skeletal muscle development. Taken together, these findings indicate that mHRC expression is not restricted to adult muscle fibers, suggesting a role for mHRC in addition to regulating Ca^{2+} concentrations during adult muscle fiber contraction.

The first approach taken to examine other biological roles for mHRC was to determine its subcellular localization in the absence of a sarcoplasmic reticulum. This localization was compared to that known for calsequestrin and calreticulin. Calsequestrin does not have a KDEL sequence but has been localized to dense vacuoles within the ER when overexpressed in myoblasts [26,27]. It is hypothesized that these vacuoles represent the genesis of a sarcoplasmic reticulum. In contrast, the ubiquitously expressed calreticulin has a KDEL sequence, localizes to the ER and does not form dense vacuoles when overexpressed in myoblasts. A myc-epitope-tagged mHRC was transfected into P19 cells (data not shown) and myoblasts (Fig. 5) and examined by immunofluorescence. In both of these cases, the majority of mHRC protein, which does not have a KDEL sequence, exhibited a perinuclear expression pattern similar to the diffuse pattern found for calreticulin. Therefore, mHRC is similar to calreticulin in the diffuse pattern of ER expression even though calreticulin has a KDEL sequence and mHRC does not. Localization to the perinuclear region is consistent with a role for mHRC in regulating cytosolic Ca^{2+} concentrations by binding Ca^{2+} in the lumen of the ER.

The calcineurin/NFAT pathway has been implicated in the

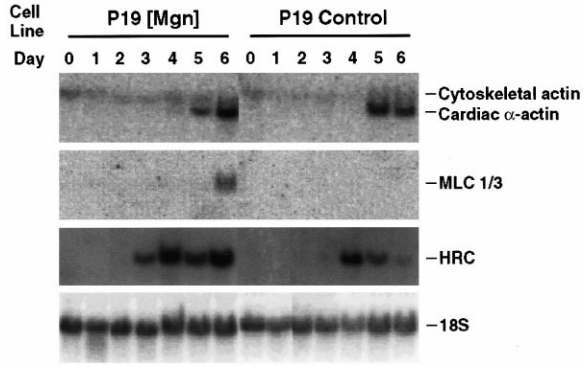

Fig. 4. HRC is expressed early during skeletal and cardiac muscle development in P19 cells. Identical Northern blots containing total RNA from a time course of P19 cell differentiation (see Section 2) were probed with α-cardiac actin, MLC1/3, mHRC and 18S as indicated.

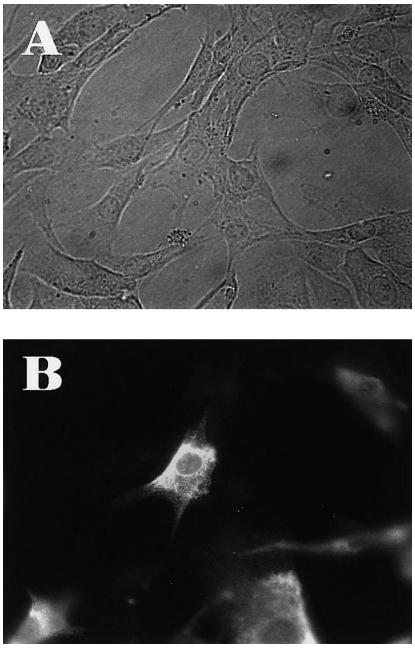

Fig. 5. HRC protein is perinuclear in myoblasts prior to terminal differentiation. C2C12 myoblasts were transiently transfected with a myc-tagged mHRC. (A) shows a phase contrast view of the transfected cells. (B) shows the perinuclear localization of HRC in a transfected cell using a monoclonal anti-myc antibody (9E10).

regulation of muscle-specific gene expression [10,11]. Since this pathway is sensitive to cytosolic Ca^{2+} levels, the ability of mHRC to affect gene expression was examined. mHRC was overexpressed in P19 cells and myoblasts in an attempt to modulate their differentiation programs. Differentiation was monitored by cardiac α-actin transcript levels and by counting colonies containing myotubes. No level of overexpression of mHRC resulted in a significant, reproducible change in the extent of differentiation of either P19 cells or myoblasts into cardiac or skeletal muscle, respectively (data not shown). In contrast, the overexpression of calreticulin inhibits the retinoic acid-induced differentiation of P19 cells into neurons [7], while overexpression of calsequestrin in transgenic mice results in the induction of a fetal gene expression program and cardiac hypertrophy [8,9]. Furthermore, mice lacking calreticulin are not viable and are defective in their cardiac development [28]. Subsequently, although calsequestrin and calreticulin have been shown to regulate gene expression, our results indicate no effect of mHRC overexpression. It is possible that the loss of mHRC expression via targeted gene disruption would reveal a biological role for mHRC not observed in the overexpression studies presented here.

In summary, mHRC has an intriguing tissue distribution suggestive of a role in the control of gene expression during cell proliferation/differentiation. Subcellular localization studies show that mHRC is present in the perinuclear region, consistent with a role in the regulation of Ca^{2+} stores. However, overexpression of mHRC during cardiac and skeletal muscle development did not result in a significant acceleration or inhibition of cardiogenesis or myogenesis. Therefore, if mHRC plays a role in the regulation of muscle-specific gene expression, it is neither rate-limiting nor inhibitory.

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